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TITLE: Dysregulated microRNA Activity in Shwachman-Diamond Syndrome

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14. ABSTRACT

Shwachman-Diamond Syndrome (SDS) is an inherited bone marrow failure primarily affecting myeloid development. Because the affected cells are rare and heterogeneous, the altered genetic networks in vivo remain unknown. The central goal of this grant is to define transcriptional signatures of bone marrow failure in SDS using single cell RNA-seq of patient cells. We will analyze these datasets to test the novel hypothesis that reduced microRNA activity contributes to hematopoietic dysfunction in SDS. To date, we have sequenced ~300 hematopoietic stem and progenitor cells (HSPC) from normal donors and SDS patients and established, to our knowledge, the first hematopoietic ontogeny at single cell resolution. Differential gene expression analyses between normal and SDS cells revealed cell-type restricted gene expression changes in every subpopulation of HSPC. Our preliminary results suggest that closely related HSPC subpopulations are variably affected by SBDS mutations, which may contribute to complex and unstable hematopoietic symptoms in patients. Ongoing and future work includes 1) annotation of differentially expressed genes to hematopoietic phenotypes in cellular and animal models of SDS, 2) targeted single cell RNA-seq to generate quantitative expression data for a panel of low abundance, disease-relevant genes that were impossible to detect using traditional RNA-seq and 3) generation of microRNA expression profiles from HSPCs to be overlaid onto mRNA profiles.

15. SUBJECT TERMS
Single cell RNA-seq; bone marrow failure; hematopoiesis; myelopoiesis; targeted RNA-seq

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Introduction

Patients with Shwachman-Diamond Syndrome (SDS) suffer from hematopoietic abnormalities including neutropenia, thrombocytopenia, pan-anemia and myelodysplasia (MDS) with progression to acute myeloid leukemia (AML). The affected cell types and altered genetic networks *in vivo* remain unknown, primarily due to the rarity and heterogeneity of bone marrow progenitors. Our overarching goal is to define transcriptional signatures of bone marrow failure in SDS by performing single cell RNA sequencing (RNA-seq) on freshly isolated patient cells. These studies may lead to the development of targeted molecular therapies as an alternative to bone marrow transplant for the treatment hematopoietic dysfunction in SDS.

Keywords

Single cell RNA-seq; bone marrow failure; hematopoiesis; myelopoiesis; targeted RNA-seq

Accomplishments

What were the major goals of the project?

Aim 1. Define the molecular basis for the SDS myelopoiesis defect at single cell resolution.

Sub-Task 1A: Consent 6 SDS patients under protocol 10-02-0057, and 6 normal donors under protocol 09-04-0167; obtain patient bone marrow samples, purify CD34+ cells. (Start date 9/30/2014; duration 24 months; protocol approved 10-16-13 and 1-27-14, respectively, by local IACUC).
Milestone: Obtain 6 patient and 6 donor samples.
Percent completion: 50%

Sub-Task 1B: Load purified cells onto C1 chips; prepare ~96 single cell RNA-seq libraries per sample; run sequencing reactions. (Start date 9/30/2014; duration 24 months)
Milestone: Generate genome-wide transcriptional profiles of CD34+ cells form normal donors and SDS patients at single cell resolution.
Percent completion: 50%

Sub-Task 1C: Process data; perform bioinformatic analyses. (Start date 3/30/2015; duration 30 months)
Milestone: Identify SDS affected progenitor cells; Define SDS gene expression networks in single cells or subpopulations of CD34+ cells; Predict microRNA-targeted mRNAs that explain pathogenesis of SDS.
Percent completion: 20%

Aim 2. Functionally annotate SDS transcriptomes to myelopoiesis defects.

Sub-Task 2A: Obtain 2-4 frozen mononuclear cells from SDS patient repository under protocol 10-02-0057 and 2-4 normal donors under protocol 09-04-0167. (Start date 9/30/2015; duration 24 months; protocol approved 10-16-13 and 1-27-14, respectively, by local IACUC).
Milestone: Obtain 2-4 SDS patient and 2-4 donor samples.
Percent completion: 0%

Sub-Task 2B: Culture CD34+ cells; over-express or knockdown key central and peripheral node

genes in SDS networks; assess myeloid/neutrophil phenotypes in myelopoiesis culture ex vivo. (Start date 9/30/2015; duration 24 months

Milestone: Identify SDS myeloid phenotypes ex vivo; define genes responsible for myeloid phenotypes; functionally annotate microRNA-dependent and -independent pathways explaining SDS pathogenesis.

Percent completion: 0%

Sub-Task 2C: Quantify microRNA activity in SDS myeloid progenitor cells; perform microarray Expression profiling of microRNAs in SDS myeloid progenitor cells; re-evaluate microRNA target predictions accounting for microRNA expression changes in affected SDS progenitor cells. (Start date 9/30/2016; duration 12 months)

Milestones: Quantify microRNA activity in SDS myeloid progenitors; discern SDS pathways that are affected by reduced microRNA activity and/or altered microRNA expression

Percent completion: 0%

What was accomplished under these goals?

Patients with SDS suffer from complex and unstable hematopoietic defects. Neutropenia is most common in early stages of the disease, with some patients progressing to pan-anemia, thrombocytopenia or MDS/AML. Due to the complexity of clinical presentation and the rarity and heterogeneity of bone marrow progenitors, the affected cell types and altered genetic networks *in vivo* remain unknown. We proposed to leverage emerging single cell RNA-seq technology to analyze bone marrow progenitors from SDS patients enrolled in a longitudinal study with our collaborators at Children's Hospital Boston.

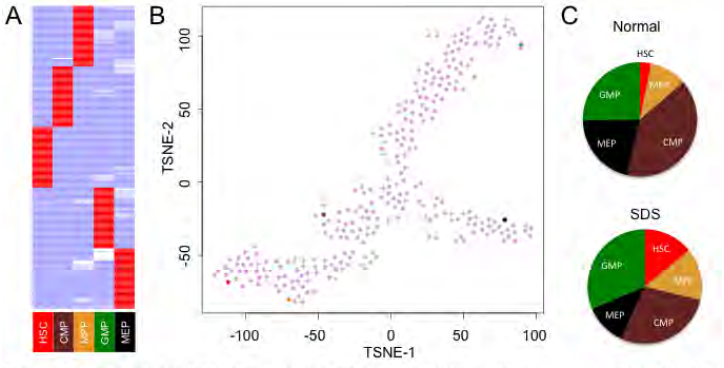

Figure 1. Building a hematopoietic ontogeny at single cell resolution. (A) We identified an expression signature from bulk RNA-seq of purified CD34+ subpopulations, comprising ~50 genes exclusively expressed in each subpopulation. (B) Non-linear principal component analysis (tSNE) was applied using this signature. Bulk samples separate as expected based on the established hematopoietic ontogeny, with single cells from normal (grey) and SDS (purple) bone marrow falling along a continuum of differentiation. (C) Model based clustering revealed an increased percentage of SDS cells in the HSC cluster.

To date, we have sequenced CD34+ hematopoietic stem and progenitor cells (HSPC) from two normal donors (n=95 cells) and four SDS patients (n=176). Unsupervised clustering of these cells did not effectively separate normal from SDS, leading us to hypothesize that disease signatures may be masked by population substructure within the CD34+ population. To remove this potentially confounding factor, we established a hematopoietic ontogeny at single cell resolution using a panel of 250 genes that were highly predictive of membership in established CD34+ subpopulations (Fig. 1A). Normal and SDS single cells fell along a continuum of differentiation (Fig. 1B), so we used model based clustering to assign cells to clusters corresponding to HSC, MPP, common myeloid progenitor (CMP), megakaryocyte-erythroid progenitor (MEP) and granulocyte-monocyte progenitor (GMP) identity. We note an increased proportion of SDS cells in the HSC cluster (Fig. 1C) which may indicate a differentiation block or compensatory increase in HSC number; however, this finding needs to be confirmed in a larger number of cells. Within-cluster differential gene expression analyses between normal and SDS cells revealed significant gene expression changes in every cluster, although many of the altered genes and pathways were different. Our data suggest that closely related HSPC subpopulations are variably affected by *SBDS* mutations, which may

contribute to complex and unstable hematopoietic symptoms in patients. We are currently working to annotate differentially expressed genes and networks to hematopoietic phenotypes in cellular and animal models of SDS using approaches described in Aim 2.

We have obtained important insights using single cell RNA-seq that we are currently validating and plan to publish in early 2016. A tentative title for this manuscript is "Single cell transcriptional signatures identify hematopoietic defects in patients with Shwachman-Diamond Syndrome". However, global transcriptome profiling at the single cell level is plagued by data quality and cost issues. For this reason, over the last year we have elected to freeze ~3000 single cell lysates from additional SDS patients and normal donors while we identified a more robust technology. Indeed, a commercial assay was recently developed for targeted RNA-seq which has proven sensitivity to quantify expression of up to 160 low abundance mRNA species from a single cell (*Precise Assay*, Cellular Research, Palo Alto, CA). *Precise* datasets will be an excellent complement to our existing datasets because they are rich in disease-relevant genes relative to global RNA-seq. Another advantage is the significant cost reduction which will enable us to profile thousands of cells, rather than hundreds as we originally proposed, offering major gains in statistical power. We expect to begin data collection using *Precise Assays* within the next month, with computational analyses extending over the next 6-12 months.

What opportunities for training and professional development has the project provided?

A graduate student in the lab, Mr. Adam Brown attended the 3rd Annual Single Cell Analysis Investigators Meeting sponsored by the NIH Common Fund (April 2015). A postdoctoral fellow in the lab, Dr. Cailin Joyce attended two meetings in the past reporting period: Gordon Research Conference *"Post-transcriptional gene regulation"* (July 2014) and Keystone Conference *"Hematopoiesis"* (February 2015).

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

In the next reporting period, we will perform targeted RNA-seq with *Precise Assays* in ~3000 cells from SDS patients (n=5, including 2 patients that were sampled previously) and normal donors (n=2). This second round of sequencing will increase our statistical power and hone in specifically on disease-relevant genes. We will initiate Aim 2 experiments with the goal of annotating differentially expressed genes and networks to SDS phenotypes using primary CD34+ cells and induced pluripotent stem cell lines derived from study patients. Additionally, we are collaborating with Dr. David Scadden's lab (MGH) to annotate genes and networks *in vivo* using their inducible Mx-Cre SBDS knockout mouse. We have added Dr. Scadden as a new unpaid collaborator and note that all animal experiments will be performed solely by the Scadden lab under their existing protocols. Finally, we will generate microRNA expression profiles from normal and SDS HSPCs that will be mapped onto mRNA expression profiles to test the novel hypothesis that microRNA activity is reduced in SDS.

Impact

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

Changes/Problems

Changes in approach and reasons for change

1) As noted above, we and others have struggled with data quality and cost issues related to global RNA-seq of single cells. Our most significant issue is that many regulators of hematopoiesis are not reliably detected by single cell RNA-seq due to low abundance. The limiting factor appears to be efficiency of reverse transcription so deeper sequencing is unlikely to solve this problem. We have now identified *Precise Assays* (Cellular Research, Palo Alto, CA) for targeted RNA-seq as the best-in-class approach for rare transcript detection in single cells, and will proceed with targeted (rather than global) RNA-seq on the 3000 single cell lysates we have banked this year. The resultant datasets will be complementary to the global datasets we have already generated.
2) We have acquired a new unpaid collaborator to replace Chris Mason on computational analyses. GC Yuan is a colleague at Dana-Farber Cancer Institute with expertise in ordering individual cells throughout the course of a biological process such as hematopoietic differentiation. His specialized skill set is uniquely suited for the needs of this project.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report.

Changes that had a significant impact on expenditures
None of the changes described above will result in >25% change in budget allocation.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

Products

Publications, conference papers, and presentations
CE Joyce, S Li, I Hofmann, C Nusbaum, C Sieff, C Mason, CD Novina. (2015) Comprehensive transcriptomic analysis of Shwachman-Diamond Syndrome at single cell resolution. Poster, Keystone Hematopoiesis.

Journal publications
Nothing to Report.

Books or other non-periodical, one-time publications

Nothing to Report.

Other publications, conference papers, and presentations
Nothing to Report.

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and/or licenses
Nothing to Report.

Other Products
Nothing to Report.

Participants and Other Collaborating Organizations

What individuals have worked on the project?

Name:	Carl Novina, M.D. , Ph.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.0
Contribution to Project:	Dr. Novina is responsible for the conception and design of all the studies. He will supervise their execution, analyze data, prepare publications related to this work, and present the findings to the scientific community.
Funding Support:	N/A

Name:	Frank Buquicchio
Project Role:	Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	10 Calendar Months
Contribution to Project:	Mr. Buquicchio worked on methods to purify and characterize RNA.

Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes. Please see Appendix A – Other Support.

What other organizations were involved as partners?

Organization Name: Boston Children's Hospital
Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Organization Name: Broad Institute of Harvard and MIT
Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Organization Name: Joslin Diabetes Center
Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Organization Name: Massachusetts General Hospital

Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Special Reporting Requirements

None.

Appendices

Appendix A – Other Support

Appendix A

OTHER SUPPORT

NOVINA, Carl D.

ACTIVE

(THIS AWARD)

BM130070 (Novina) 08/15/2014-08/14/2017 0.48 CM
DOD
Dysregulated microRNA activity in Shwachman-Diamond Syndrome
Specific Aims: (1) Define the molecular basis for the SDS myelopoiesis defect at single cell resolution; (2) Functionally annotate SDS transcriptomes to myelopoiesis defects

(NEW)

1 DP1 DK105602-01 (Novina) 09/18/2014-08/30/2019 6.12 CM
NIH
Engineering epigenetic therapy for sickle cell disease
The goal of this project is to engineer site-specific DNA binding module fusions with DNA demethylating enzymes for epigenetic induction of fetal hemoglobin (HbF) for therapy of sickle cell disease (SCD).
The DP1 program does not have Specific Aims

5R01 DK102165-02 (Novina) 09/29/2013-09/28/2017 0.93 CM
NIH
"Dysregulated microRNA function in Diamond Blackfan Anemia"
Specific Aims: (1) Comprehensive definition of the molecular basis for the erythropoiesis defect in DBA using bone marrow aspirates and induced pluripotent stem (iPS) cells; (2) Functional annotation of DBA transcriptomes and translatomes to erythropoiesis defects.

5R01 CA140986-04 (Novina) 09/01/2011-07/31/2016 0.93 CM
NIH
"Analysis of cap-dependent translational repression by microRNAs in oncogenesis"
Specific Aims: (1) Determine the effect of eIF4E perturbation on cancer-relevant changes in melanomas; (2) Identify and functionally validate miRNA-targeted mRNAs affected by perturbing eIF4E in melanomas

9617648 (Novina) 02/01/2013-01/31/2016* Research Expenses Only
Dana-Farber Cancer Institute
Claudia Adams Barr
"Networking NFAT5 targets for melanoma therapy"
The goal of this project is to develop innovative tools that will broaden the use genome editing technologies and will transform experimental approaches to discovering roles for genetic changes detected in cancers.
Specific Aims: (1) Develop physiological approaches to study gene function in human cancer cells; (2) Systematically define melanoma transcriptional targets of NFAT5 by ChIP-seq; (3) Map NFAT5-regulated networks and annotate targets in melanoma metastasis
**First one year no cost extension*

1R01 CA185151-02 (Novina) 05/21/2014-04/30/2018 2.79 CM
NIH
(PQD1) Evolution of vemurafenib resistance in circulating melanoma cells
The overall goal of this project is to identify and annotate altered transcriptional networks that arise in melanoma circulating tumor cells (CTCs) during the evolution of drug resistance.
Specific aims: (1) Generate transcriptional profiles from melanoma CTCs; (2) Determine pathways of vemurafenib resistance; (3) Restore vemurafenib sensitivity in human melanoma cells by modulating CTC resistance pathways.

6240101 (Novina) 08/01/2013-07/31/2016* 0.08 CM*
American Society of Hematology, Bridge Grant
Dysregulated microRNA function in diamond blackfan anemia
Specific Aims: (1) Comprehensive definition of the molecular basis for the erythropoiesis defect in DBA using bone marrow aspirates and induced pluripotent (iPS) cells; (2) Functional annotation of DBA transcriptomes and translatomes to erythropoiesis defects.
**Second one year no cost extension*

(NEW)
Project Number N/A (Ostermeier/Novina) 04/01/2015-03/31/2018 0.30 CM
National Science Foundation
Collaborative Research: Targeted CpG Methylayion
Specific Aims: (1) Create modular, targeted cytosine MTases capable of achieving >95% methylation at a desired target site with undetectable methylation at non-target CpG sites (Ostermeier); (2) Develop a scalable screening pipeline for defining repressive CpG methylation in eukaryotic promotors (Novina).